# Fabrication of a microfluidic flow-through immunoassay for simultaneous detection of multiple proteins

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## Abstract

We have developed a chip-based microfluidic device for multi-analyte immunoaffinity capture and detection of proteins. The immediate motivation is an epidemiological study of the immune response to the Human Papilloma Virus (HPV), for which the simultaneous isolation and detection of multiple proteins from a large number of microliter samples of cervical secretions is required. Using the microfabrication facilities at NIST, we are able to make micrometer-scale glass-encapsulated microfluidic systems with any desired two-dimensional configuration. The prototype devices consist of a long glass-encapsulated channel, 50$\mu$m x 15$\mu$m x 30cm, with a serpentine pattern. Side ports are used for electroosmotic loading of different biotinylated antibodies into each segment of the channel. These antibodies bind to streptavidin that has been covalently bonded to the channel walls via an imine linkage. The robust attachment of the antibodies allows them to be used for multiple sample runs; after each run, the antibody-antigen interaction can be disrupted by an acidic buffer gradient, leaving the tethered antibodies ready for reuse. After the antibodies have been immobilized, the sample under analysis flows through the entire device. Electrical control of the sample flow permits adjustment of the residence time in each segment in order to optimize binding. The channel device architecture has several advantages over existing array technology: the proteins are detected by single-point capture, and much smaller sample volumes can be used.

## Overview and objectives

**Why microfluidics?**

**Smaller** – System volumes less than a microliter are possible, allowing detection of smaller amounts of analyte.

**Faster** – Diffusion-mediated processes happen more quickly in a smaller system. Multiplexing measurements is more easily accomplished.

**Cheaper** – With smaller systems, use of expensive reagents (e.g., capture ligands) is greatly reduced.

**Develop microfluidic expertise in-house**

Focus on specific applications that require extensive customization or integration into a measurement system.

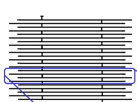
**Initial goal** - develop microfluidic device for immunoaffinity analysis of small samples

Simultaneous detection of multiple analytes using specific binding interactions.

With a system volume of 1$\mu$L, detection of 10pg/mL concentration → sub-picogram detection capability

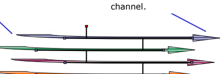
Integrate with optical detection instrumentation.

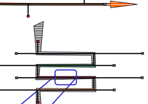
## Device operation - schematic



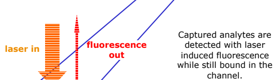
Serpentine channel pattern for flow-through immunoassay

Using the side access ports, a different capture antibody is loaded into each channel.





As the sample flows through the device, analytes are captured by the immobilized antibodies.

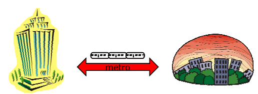



Captured analytes are detected with laser induced fluorescence while still bound in the channel.

The device can be **reused** after eluting the bound proteins with a dissociating agent (e.g., acidic buffer).

## Interagency collaboration

This area is ideally suited for a collaboration between researchers at NIST and NIH. Microfluidics is an active area of research at NIST Gaithersburg. Device fabrication typically employs techniques and equipment first developed for the semiconductor industry. These facilities, and the technical expertise to operate them, already exist just up the road from NIH.



NIH

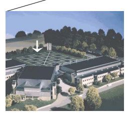
Clinical and research applications

Wide experience with biological systems

NIST

Fabrication facilities

Microfluidics expertise

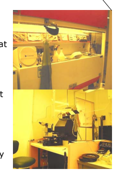


New cleanroom facility at NIST, being furbished now.

Sections of the cleanroom currently in use at NIST.

Top: wet etch bench and resist spinners

Bottom: mask aligner for photolithography



## Initial strategy

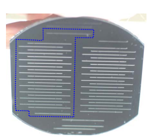
Initial devices are made from **silicon/glass**:
- system and fabrication techniques are well-characterized.
- chemistry for biomedical applications better understood.
- easier to incorporate circuit elements in future applications.

Control flow with both **pressure** and **voltage**:
- pressure-driven flow (vacuum) for initial chemistry
- electrokinetic flow of sample through device permits better control, less dilution of sample plug during measurement.

Attach antibodies with **avidin-biotin chemistry**:
- antibodies strongly linked to surface so devices can be reused
- well-established and widely used chemistry

Detect bound molecules with LIF **inside channels**:
- laser-induced fluorescence of tagged proteins after single-point capture
- detecting proteins bound in channels gives the most signal, but potentially higher background.

## Prototype device

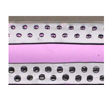


Prototype device has twenty 1-cm long channels.

Two devices per 3" wafer, with full optical access on one side

Fluid access to ports is through silicon wafer

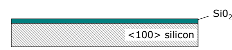
Strips of PDMS confine aqueous buffers to individual ports



## Making glass-encapsulated channels

Starting material:

3" diameter silicon wafers with oxide
Techniques for silicon processing are well-characterized
Future possibility of adding on-chip electrical elements



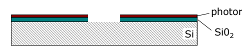

Patterning the channels:

Wafers are coated with photoresist, which is easily removed (developed) after exposure to light.

During illumination, only the channel area is uncovered.

After developing, the exposed oxide is removed with hydrofluoric acid.

Minimum feature size is determined by wavelength of light: typically 0.5-1$\mu$m.

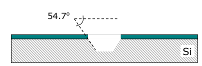



Forming the channels:

Oxide layer serves as a mask for silicon etch

**Anisotropic etch:** TMAH etches preferentially perpendicular to the <100> Si planes, leading to a trapezoidal cross-section.

Typical channel dimensions: 50 $\mu$m wide, 15 $\mu$m deep, 30 cm long.




Coating channel with uniform glass layer:

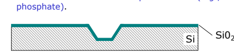
After etching, grow a thick layer of thermal silicon dioxide.

Chemistry for functionalizing $SiO_2$ surfaces is well-established.

Anodic bonding to glass wafers has been successful with oxide layers as thick as 650 nm.

Estimated breakdown voltage ~ 500 V dry.

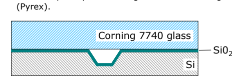
Measured breakdown voltage > 400V (substrate positive) when channel is filled with aqueous buffer (e.g., 20mM phosphate).




Sealing the channel:

Permanently attached with anodic bonding: sandwich heated to 400 C, voltage of 1200 V applied for 30 minutes.

Glass used: optically flat Corning 7740 borosilicate glass (Pyrex).

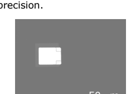



## Connecting to the channels

Lithographic patterning of the through-holes allows small-volume ports with good control of both geometry and surface quality.

Requires the capability to align the back and front side patterns with better than 1$\mu$m precision.


Reflected light




Transmitted light

Pictures are glass-side up. Estimated port volume: < 6 pL

For pressure-driven flow, use Nanoports (Upchurch) or vacuum.

For electrokinetic flow, build PDMS reservoirs on top of silicon for aqueous buffers. PDMS sheets can also be used to seal unused ports (at negative or atmospheric pressure).

## Tethering Antibodies in Microchannels

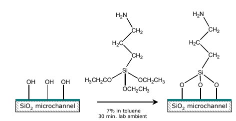
This device requires a robust and general method for tethering antibodies to the microchannel walls. Using a streptavidin-biotin linkage has several advantages:

Biotinylated antibodies are available commercially.

Attachment of biotinylated antibodies to the streptavidin-coated surface is the same for different antibodies

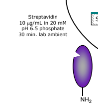
Attached antibodies are positioned off of the surface, with functional antigenic binding sites accessible.

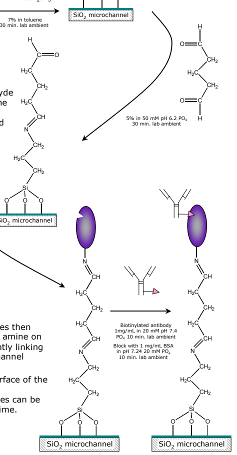
But first, the streptavidin must be covalently linked to the surface.




An aminosilane reacts with the glass microchannel surface to yield reactive primary amines

1,5-pentanedialdehyde is linked to the amine groups, providing a aldehyde-terminated surface.

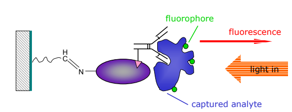



The aldehyde moieties then react with a primary amine on streptavidin, covalently linking the protein to the channel surface.

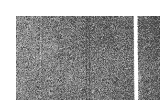
At this stage, the surface of the device is stable, and biotinylated antibodies can be added one leg at a time.

## Detection of bound analytes



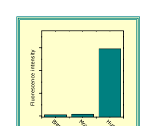

The fluid sample is mixed with a fluorophore (e.g., Alexafluor 633, Cy5) that binds to all proteins (amine groups)

The surface-bound antibodies capture the fluorescently-tagged complementary antigens; only areas of the channel with captured protein are fluorescent.



Recognition with minimal nonspecific binding:

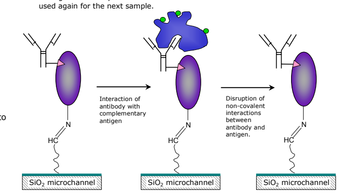
Silicon microchannels terminated in anti-human IgG were exposed to mouse IgG, rinsed, and then exposed to human IgG. Protein concentration was approximately 1 nM.



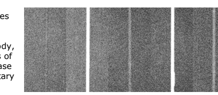

## Reusing the capture antibodies

Releasing captured antigens

The interactions between the antigen and the antibody can be disrupted by rinsing with a mildly acidic buffer. The surface-bound antibodies can then be used again for the next sample.

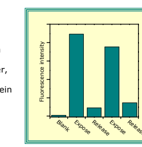



Fluorescence images of a silicon/glass microchannel with immobilized antibody, through two cycles of exposure and release of the complementary antigen

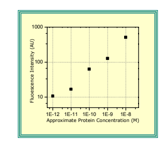


Reuse of capture antibodies:

Silicon microchannels terminated in anti-human IgG were exposed to human IgG, rinsed with acidic buffer, exposed again to human IgG, and rinsed again. The approximate protein concentration used was 1 nM.




## Towards quantitative detection



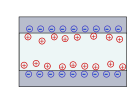

Measured fluorescence varies with analyte concentration:

Silicon microchannels terminated in anti-human IgG were exposed to increasing concentrations of human IgG.

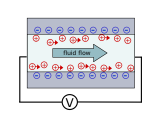
Current limit of detection ~ 10 pM (1.3 ng/ml) using a microscope with halogen lamp and CCD camera.

## Controlling fluid flow

The flow of the sample through the serpentine channel pattern is achieved with **electroosmotic flow**:



Fixed charge on the interior walls of the microchannels attract counterions from the buffer to the sidewalls.



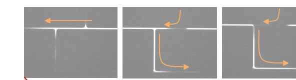

When voltage is applied along the channel, the electric field pushes these counterions. The moving ions drag nearby solvent molecules along move, causing bulk flow in the microchannel.

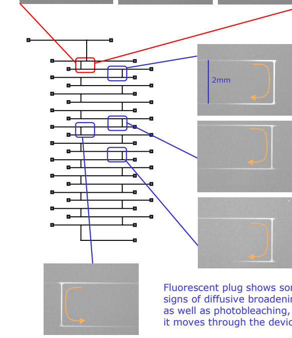
By changing the voltages applied to the different fluid reservoirs at different times, a small plug of fluid can be injected into the device.

## Tracking fluid flow

Orange arrows indicate direction of flow for two stages of sample injection.

Fluid flow in the prototype device is monitored using sulforhodamine B in 20 mM phosphate buffer, pH 7.4.






Fluorescent plug shows some signs of diffusive broadening, as well as photobleaching, as it moves through the device.

After injection, the fluorescent plug can be driven through the serpentine channel. Monitoring the flow of a biological sample, without photobleaching the fluorophore-tagged proteins, should be possible using a longer-wavelength tracer.

Flow through the 30-cm device takes approximately 30 minutes.

## Current status

**Device fabrication:**

Made prototype devices with glass-encapsulated channels and lithographic back-side ports.

Arrays of straight channels permit rapid parallel tests of attachment chemistry.

**Flow control:**

Have shown independent control of flow in different channels.

Have demonstrated both pressure-driven and electro-osmotic flow in these channels.

Electrical control of flow permits detailed control of injected sample plug.

EOF mobilities: bare $SiO_2$ $\mu_{EOF}$ = 3.5x10$^{-4}$ cm$^2$/V-s.
aminated channel $\mu_{EOF}$ = -1.7x10$^{-4}$ cm$^2$/V-s.

**Attachment chemistry:**

Robust tethering of antibodies to microchannel surfaces.

Molecular recognition of complementary antigens with minimal non-specific interactions.

Demonstrated reuse of immobilized capture antibodies.

## Future directions

**Short-term goals:**

Quantitative characterization of functionalized surfaces.
Further optimize quantitation of analyte capture.
Begin reliability/reusability testing.
Integration into whole-chip detection system.

**Long-term possibilities:**

Incorporate additional on-chip functionality, such as detection hardware or labeling chemistry.
Explore the use of plastics for biomedical applications of microfluidics.
Other clinical and research applications.